Published in final edited form as:

Curr Opin Cell Biol. 2017 August ; 47: 92–98. doi:10.1016/j.ceb.2017.03.010.

The coordination of membrane fission and fusion at the end of autophagosome maturation

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Abstract

The two major objectives of macroautophagy are to sequester cargo away from the cytoplasm and deliver this material for breakdown in the lysosome. Sequestration is complete when the autophagosome membrane undergoes fission to produce separate inner and outer membranes, while delivery into the lysosome requires fusion of the outer autophagosome membrane with the lysosome membrane. Thus, the merging of membranes through fission and fusion underlies each of the pivotal events in macroautophagic clearance. How these merging events are controlled in the cell is poorly understood. Several recent studies however suggest that the two events may be temporally coordinated and rely upon members of the classic membrane fusion SNARE family as well as the autophagy-specific family of Atg8 proteins.

Graphical abstract

Keywords

membrane fusion; membrane fission; Atg8; Syntaxin 17

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The final stages of autophagosome maturation involve two membrane merging events

Macroautophagy begins with the formation of a bowl-shaped structure called the isolation membrane that grows and engulfs portions of the cytoplasm (Figure 1). Eventually, the rims of the bowl come together, sealing the captured cytoplasm inside a double membrane vesicle called the autophagosome. Although closure of the autophagosome is an area of heavy interest, we still have only a vague understanding of the mechanism and the factors that contribute to this event. Topologically, closure is a constriction of a hole followed by a membrane fission event that produces separate internal and external vesicles. Thus, this process may utilize ESCRT proteins (Figure 1) in much the same way ESCRTs drive inward vesicle budding and membrane fission at multivesicular bodies. Indeed, ESCRT proteins are needed to support autophagic flux [1–3], and depletion of ESCRT proteins leads to an accumulation of open isolation membranes in Arabidopsis [4]. Nonetheless, direct involvement or clear localization of ESCRTs at the autophagosome membrane has not been demonstrated.

Alternatively, the Atg8 family is also closely associated with autophagosome membrane dynamics. Atg8, and its mammalian homologs LC3 and GABARAP, become covalently linked to phosphatidylethanolamine (PE) on the developing autophagosomal membrane (for review [5]). Yeast expressing little [6] or no Atg8 [7, 8] form abnormally small autophagosomes, suggesting these proteins regulate membrane expansion. Mammalian cells lacking either LC3 or GABARAP also have problems in membrane expansion, and accumulate more open isolation membranes [9], suggesting a role in autophagosome closure. Open membranes also accumulate if Atg8 lipidation is blocked [10] or is rapidly reversed [11]. Thus, Atg8 family proteins regulate autophagosome maturation including possibly the closure/fission of the mature autophagosome.

Closed autophagosomes must then fuse with the lysosome to degrade the captured cargo. This event is topologically equivalent to other vesicle trafficking activity in the cell and not surprisingly is governed by SNARE fusion machinery. But how SNAREs are recruited to the growing autophagosome is unusual ([12] and discussed below) and the timing has suggested recruitment may be sensitive to changes in the autophagosome membrane shape or lipid composition.

Now a series of papers suggest that fusion into the lysosome is coordinated with autophagosome closure, ensuring that the cargo-laden inner vesicle is separate from the outer membrane before lysosome engagement. Atg8 proteins function at both steps, accelerating membrane fission [13, 14] and coordinating the fusion apparatus [14]. After fission, cues on the autophagosome and possibly the lysosome promote SNARE insertion on the closed autophagosome [13, 15]. Interestingly, different macroautophagic cargoes are associated with different SNARE families [14, 16] and thus future work will have to establish whether tight fission/fusion coordination is conserved for all autophagosome SNARE-mediated events or is unique to classic starvation and basal autophagy pathways.

The case for Atg8 protein involvement in membrane fission

To assess how Atg8 family proteins impact membrane fission during macroautophagy, Nguyen and co-workers used CRISPR-Cas9 to knockout all six Atg8 family members in HeLa cells[14]. They then poisoned cells to drive the autophagy-dependent clearance of dying mitochondria. In the absence of Atg8 proteins, mitochondrial turnover fails but the cell still manages to package defective mitochondria into autophagosomes. Both electron tomography of thin-sectioned samples and protease protection on isolated lysates confirm that these autophagosomes are fully sealed. Thus, although delivery to the lysosome is disrupted, Atg8-deleted cells still complete the closure of autophagosomes by membrane fission. And like earlier studies with low expression of Atg8, the knockouts are also defective in membrane expansion.

Although Atg8 knockouts still produced closed autophagosomes, the actual sealing takes significantly longer (6 hours vs. < 2 hours). Ideally, one could localize the kinetic impairment to the fission step or an earlier membrane growth event by following the transitions with live imaging. Unfortunately, the fission-dependent separation of inner and outer membranes, has not been resolvable by light microscopy. Now, Tsuboyama and colleagues, suggest autophagosome fission might be detectable as a subtle morphological change [13]; Specifically, the authors note that starvation-induced growing autophagosomes are typically elliptical, but at a very late stage of maturation (most likely when fission occurs) these compartments suddenly adopt a spherical structure. Intriguingly, in LC3 lipidation defective cells, the authors found the conversion from elliptical to spherical occurred much more slowly, with some rare samples even reverting to fully open cup-like shapes. Thus, both groups detect a slowing of the rate of autophagosome fission when lipidated Atg8 proteins are missing, but consistent with many other groups [7, 8, 17], there is no absolute requirement for these proteins. This suggests that perhaps a principal role of the Atg8 family is to accelerate the fission event.

How might Atg8 proteins catalyze membrane fission?

In vitro reconstitution studies on lipid-anchored Atg8 proteins have suggested that they are membrane-active. Each can tether liposomes together into clusters [18–24], and in some cases, support the mixing of lipids as either hemifusion or full fusion of lipid bilayers. Importantly the sequences that support these *in vitro* activities are also essential in cells. On the other hand, the energetic bar to membrane fusion is often lowered artificially *in vitro* by using unstable liposomes. When efforts have been made to use stable lipid mixtures that are low in conical shaped lipids [21, 22] or when membranes are not under curvature-induced strain [25, 26], lipid-mixing is not observed even though membrane tethering persists.

Could tethering be enough to support fission? To tether two apposing membranes, lipid-anchored Atg8 must either bind the lipids, or proteins (presumably another copy of Atg8) on the target bilayer (Figure 2). Combinations of biochemistry and structural biology suggest both occur. Weidberg and colleagues identified membrane-active peptides at the amino termini of mammalian Atg8 proteins that directly engage lipid through either hydrophobic or electrostatic interactions [19]. These peptides are essential for *in vitro* tethering and fusion

and broadly resemble fusion peptides previously identified in enveloped viruses targeting mammalian membranes. Similar results were seen in a recent highly comprehensive study of *C. elegans* Atg8 proteins [24], suggesting these activities are broadly conserved. As a putative homo-oligomeric state can be seen in some crystal-packing arrangements [27], an intriguing idea is that Atg8 also unfolds to an open state that promotes *trans*-complexes bridging two membranes (as described here [19]). However, NMR of individual GABARAPs coupled to small nanodiscs could not detect significant conformational dynamism [28]. Instead, Wu *et al* now suggest that at least in *C. elegans*, the ability to form the closed or open conformation is isoform specific and thus different Atg8 proteins adopt preferential structures [24]. Collectively, these studies suggest that Atg8 buries a portion of its amino terminus in target membranes and may form *trans* homo-dimers to support tethering *in vitro* (Figure 2).

In vivo, the topology of membrane fission is somewhat different from the *in vitro* fusion studies [29], but perhaps two key features are similar. First, if the pore can constrict to very short membrane separation distances, *trans*-complexes could help bridge a final closure event. Because Atg8 is a small mostly compact protein, Atg8-Atg8 complexes will not span the space between apposing membranes until the distance across the pore is less than ~10 nm (~4 nm from each Atg8 protein; Figure 2). Whether the final pore exists as a circle, or some other perhaps slit-like aperture that might better accommodate this bridging is not well described. Second, to promote lipid-mixing *in vitro*, Atg8 requires somewhat unstable membranes. Interestingly, the highly curved rim of the constricting pore is likely to be naturally unstable [30]. If Atg8 proteins were to concentrate at this site, they may support the local deformation of the membrane. Indeed, studies *in vitro* detect a modest enrichment of yeast Atg8 onto highly-curved membranes[25].

The coordination of autophagosome-lysosome fusion with autophagosome fission

Autophagosome biogenesis involves contributions from many organelles and thus, it is not surprising that SNARE membrane-fusion proteins are fundamental to autophagy. For example, the biogenesis of Atg9-positive vesicles utilizes classic trafficking protein assemblies, including specific SNAREs, Rabs, and tethering complexes (e.g. [22, 31–33]). Likewise, yeast genetics suggested autophagosome-vacuole fusion during both starvation-mediated autophagy and the cytoplasm-to-vacuole targeting biosynthetic pathway relies on SNARE machinery (e.g. [34–36]). Surprisingly however, a careful screen in yeast of candidate SNARE proteins was unable to surface SNAREs that were actually resident upon the maturing autophagosome [37]. This discovery presented a key early conundrum of autophagosome maturation: a compartment that does not have SNAREs can never fuse with new vesicles to gain SNAREs and would thus seem to be excluded from normal paradigmatic vesicle trafficking pathways.

Advances in the last five years have begun to explain this conundrum. Multiple labs working in mammalian systems identified the ER SNARE, Syntaxin 17 (STX17), as an autophagosome resident protein [12, 38] (Figure 1). Unexpectedly, STX17 is only inserted

into the highly mature autophagosomes through a mechanism that does not appear to rely upon membrane-fusion. Instead the Mizushima group has proposed that an extended glycine-rich interface between its two transmembrane domains allows STX17 to adopt a semi-soluble state which can then penetrate membranes in response to an as of yet uncertain signal [12]. Once anchored on the autophagosome, STX17 recruits SNAP-29 and pairs with VAMP8 on the lysosome to drive fusion. Like other lysosome-directed SNARE complexes, STX17-mediated fusion is regulated by both the HOPS complex and Rab7 [39, 40], and depends upon activation by its cognate SM protein, VPS33A [41].

Two recent papers now implicate both lysosome and autophagosome signals in controlling STX17 recruitment. Working in mouse embryonic fibroblasts, Hubert *et al* show that STX17 recruitment requires the lysosomal protein LAMP-2 [15]. When LAMP-2 is knocked out, induction of macroautophagy still drives the formation of LC3-positive autophagosomes, but STX17 recruitment fails and fusion between autophagosomes and lysosomes is lost [15]. Lysosome-related SNARE machinery including Rab7 and VAMP8 are unaffected, suggesting STX17 recruitment is not tied to the formation of a SNARE-fusion assembly site. Instead, the authors propose an indirect consequence of LAMP-2 depletion based on its ability to bind and regulate cholesterol export at the lysosome [42, 43]. One consequence of reduced available cholesterol is the formation of interorganelle complexes between the ER and both the autophagosome and lysosome. These complexes restrict organelle motility and limit autophagosome-lysosome fusion [44]. This interpretation implies that STX17 recruitment is tied directly to autophagosome-lysosome engagement.

However, the live microscopy fission assay developed by Tsuboyama *et al* also suggests that any role for LAMP-2 or lysosomes must be indirect; their work clearly places autophagosome-lysosome docking downstream of STX17 recruitment [13]. Intriguingly, STX17 insertion almost perfectly demarcates the pre- and post-fission stages of the autophagosome [13]. LC3-positive structures are initially elliptical and STX17-negative. Immediately following (or perhaps just before) STX17 recruitment the majority of these structures adopt a spherical structure which they interpret as the closure of autophagosome following fission. STX17 recruitment is rapidly followed by lysosome docking and acidification of the autophagosome. Remarkably, the authors are also able to discriminate two acidification events within the autophagosome; the space between autophagosome membranes acidifies first, followed by full acidification of the internal vesicle as the inner membrane breaks down. Importantly, the rupture of the inner membrane coincides with the release of STX17 from this compartment. How the integrity of the inner membrane is sensed by STX17 is not yet known, but collectively the insertion and removal pathways suggest that STX17 is highly sensitive to membrane composition. Perhaps then the role for LAMP-2 is indirect, and simply reflects that the lipid composition of the autophagosome itself is altered during disrupted cholesterol homeostasis, becoming incompatible with STX17 insertion.

Proteins involved in autophagosome biogenesis, including Atg9A, FIP200 and Atg14 are all essential to the formation of STX17-decorated structures [13] and may support STX17-mediated fusion [45]. However, the recruitment of STX17, does not require any of the autophagy-specific proteins that govern the late stages of autophagosome maturation (Figure 1); loss of proteins that promote Atg8 family lipidation [13], or deletion of all six Atg8

homologs[14], does not prevent STX17 recruitment. Instead, similar to membrane fission, the speeds of both STX17 recruitment and inner membrane rupture are strongly diminished [13]. Thus, these events are likely interdependent, and suggest a role for Atg8 proteins in ensuring that these processes occur on biologically-relevant time scales.

Whether Atg8 proteins also influence the autophagosome-lysosome fusion event is still controversial. Tsuboyama *et al* [13], like other recent papers [17], detects complete autophagic delivery into the lysosome even in Atg8-lipidation deficient cells. In contrast, Nguyen *et al* [14], observe a block to autophagic flux, which can be rescued by the expression of a single Atg8 family member. In particular, re-expression of GABARAP supports the recruitment of the HOPS complex to autophagosome-lysosome interfaces. As the HOPS complex is typically associated with accelerating SNARE-mediated membrane fusion, this implies that the GABARAPs may be playing an indirect role in also facilitating the final fusion event.

The unconventional uncoupling of fission and fusion?

The two groups also differ significantly on whether STX17 is essential. STX17 appears to be required for the final fusion event during basal and starvation mediated autophagy [12, 39], but Nguyen *et al* and an earlier study find that it is dispensable for mitophagy [14, 46]. Thus, perhaps there are other SNAREs that play a role in specialized cargo delivery. A role for additional SNAREs becomes even more likely if autophagosomes are generated with predetermined destinations other than the lysosome, as occurs with the unconventional secretion of cytoplasmic molecules like IL-1 β [47, 48]. SNAREs do not only serve as the basic machinery underlying membrane fusion, but also play a pivotal role in determining the specificity of individual vesicles and target membranes [49]. Thus changes in the complement of SNARE proteins available on a vesicle will direct these vesicles to engage other targets in the cell. How secretory autophagosomes avoid the degradative fate of lysosomal fusion has been unclear, but an early example of unconventional autophagic secretion implicated the eventual targeting of specific plasma membrane SNARE proteins [50]. Codifying this SNARE-dependent specificity, Deretic and colleagues have now established that secretory and catabolic autophagic pathways are in fact dependent upon distinct SNARE machineries [16]. Most importantly, they show that STX17 is not essential to autophagic secretion of IL-1 β , and instead the R-SNARE, Sec22b decorates the autophagic membrane. Sec22b is an ERGIC compartment resident protein which can biochemically segregate with LC3-decorated compartments in an *in vitro* reconstitution of LC3 lipidation [51]. The Deretic lab now suggests that Sec22b is loaded directly onto LC3-positive compartments that are destined for secretion. These compartments engage the plasma membrane t-SNARE complex consisting of either Syntaxin 3 or Syntaxin 4 and either SNAP23 or SNAP29 [16]. The decision to integrate Sec22b appears to be coupled to the cargo receptor TRIM16 which coordinates specific cargo recognition and autophagosome biogenesis [52]. In this case the cargo, IL-1 β , is a cytoplasmic peptide that is generated by proteolytic cleavage and then pumped into the lumen of membranes destined to become autophagosomes [53]. Precisely how TRIM16 engages either IL-1 β or Sec22b remains uncertain. Likewise, whether the incorporation of SNARE proteins in these instances is also tightly connected to the fission of an autophagic vesicle has not yet been

explored. Interestingly however, because the “loading” of a cargo like IL-1 β may not require a closed autophagosome (the cargo is pumped directly into the space between the autophagosome membranes [53]) it is possible that this type of process would not require careful coordination of fission and fusion, or perhaps not generally require fission at all.

Future directions

The apparently complicated coordination of membrane fission and fusion shown in Figure 1 is arguably the most simplistic scenario. In many cases of selective autophagy where only portions of a target organelle (like the nucleus) is sequestered, fission of the cargo organelle must also be coordinated with autophagosome closure via mechanisms that are still largely unclear. In addition, many autophagosome biogenesis models suppose that the isolation membrane grows by being extruded out of a pre-existing organelle such as the ER (eg. [54]). In these cases, the fission event that closes the autophagosome must also pinch it off from the mother organelle in a process that might topologically better resemble a combination of fusion and fission. Improved assays, like those described by Tsuboyama *et al*, are needed to follow these dynamic events in real time. Likewise, each of these events is bound to depend on lipid compositions, and unfortunately the lipidomics of the autophagosome remain very poorly understood.

Acknowledgments

This work was supported by the NIH (GM1000930 and NS063973; TJM) and the China Scholarship Council/Yale World Scholars Program (SY).

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Highlights

Autophagosome closure requires Atg8s to proceed at biologically relevant speeds

Autophagosomes recruit diverse SNAREs depending upon their ultimate fate

Syntaxin 17 recruitment and autophagosome fission are temporally coordinated

Syntaxin 17 supports some, but not all, autophagosome fusion events

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Figure 1.

Membrane fission/fusion events in autophagosome maturation. Two essential membrane fission/fusion events occur during autophagosome maturation. First, membrane fission of a constricting pore closes the autophagosome and separates the outer and inner membranes into distinct structures. The machineries driving this event are still uncertain. The membranes are topologically similar to the endosomal sorting complexes required for transport (ESCRT) targets, but a number of studies including two recent works from the Mizushima and Lazarou labs suggest that the Atg8 family of proteins is actively involved in autophagosome sealing. Second, closed autophagosomes fuse with lysosomes in a classic soluble NSF attachment protein receptor (SNARE)-dependent event. STX17 is recruited to mature autophagosomes and then complexes with SNAP-29. Fusion is driven by the interaction of this complex with lysosomal VAMP8. Atg8 proteins may also regulate this event, through protein-protein interactions including the recruitment of SNARE effector proteins.

Figure 2.

Possible mechanisms for Atg8-mediated membrane tethering and fission/fusion. LC3B soluble structure (PDB: 1v49) is shown which adopts the “closed” conformation of Atg8 proteins. The bulk of the protein is the ubiquitin-like fold (colored in blue). At its amino terminus are two alpha-helices (1 in orange, 2 in red), which in the closed conformation lie near the ubiquitin core. However, other structures of Atg8 family proteins suggest they may also adopt an open conformation, perhaps in a protein isoform specific manner or perhaps as a result of other post-translational modifications including the attachment of a phospholipid at the carboxy-terminus. Lipidated Atg8 family proteins can tether and fuse membranes in vitro which might explain their role in membrane fission in vivo. Tethering and fusion rely upon direct membrane engagement of 1 with the apposing membrane (in either the closed (1) or open (2) conformation) and the homo-oligomerization of Atg8 proteins in trans, spanning the two membrane surfaces (3).